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# Subunit structure of the acetylcholine receptor from *Electrophorus electricus*

(amino acid sequence/homologous proteins/pentameric complex/receptor evolution)

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**ABSTRACT** **The amino-terminal amino acid sequences of the four major peptides ($M_r$ 41,000, 50,000, 55,000, and 62,000) present in purified preparations of *Electrophorus electricus* nicotinic acetylcholine receptor (AcChoR) have been determined for 24 cycles by automated sequence analysis procedures yielding four unique polypeptide sequences. The sequences showed a high degree of similarity, having identical residues in a number of positions ranging between 37% and 50% for specific pairs of subunits. Comparison of the sequences obtained with those of the subunits of similar molecular weight from *Torpedo californica* AcChoR revealed an even higher degree of homology (from 46% to 71%) for these two highly diverged species. Simultaneous sequence analysis of the amino termini present in native, purified *Electrophorus* AcChoR showed that these four related sequences were the only ones present and that they occur in a ratio of 2:1:1:1, with the smallest subunit ("$\alpha_1$") being present in two copies. Genealogical analysis suggests that the subunits of both *Torpedo* and *Electrophorus* AcChoRs derive from a common ancestral gene, the divergence having occurred early in the evolution of the receptor. This shared ancestry and the very early divergence of the four subunits, as well as the highly conserved structure of the AcChoR complex along animal evolution, suggest that each of the subunits evolved to perform discrete crucial roles in the physiological function of the AcChoR.**

The nicotinic acetylcholine receptor (AcChoR) is one example of a membrane protein present on the surface of excitable cells whose function is to transiently alter the ion permeability of the cell membrane in response to a neurotransmitter or a drug. The AcChoR is the only neurotransmitter receptor that has been purified to homogeneity from different animal species (reviewed in ref. 1). *Torpedo* AcChoR is a complex of four homologous subunits in a stoichiometric ratio of 2:1:1:1 (2–4). This complex unit contains both the binding site(s) for agonists and antagonists and the cation gating "structure" (5, 6) (reviewed in ref. 1). Its reconstitution into artificial membrane systems restores the physiological action of the native AcChoR both qualitatively (7–13) and quantitatively (14–16) and, in agreement with earlier results (5, 6), it was also shown (16) that the AcChoR composed of the four polypeptides is sufficient for full physiological function.

Study of the AcChoR from sources other than *Torpedo*—such as *Electrophorus* electric organ, muscle, or brain—has been hampered by difficulties in obtaining suitable amounts of intact AcChoR, due to the much lower AcChoR content of these tissues and to their high levels of protease activity. Similarities in the pharmacology, morphology, antigenicity, and physical properties (1) as well as the frequent presence upon $NaDodSO_4$ gel electrophoresis of a complex polypeptide pattern (1), reminiscent of the subunit pattern of *Torpedo* AcChoR, suggest the likelihood of close structural and functional similarities between the AcChoRs from different species. To prove the existence and the extent of such similarities between AcChoRs from different sources is crucial. It would justify the use of analogy from *Torpedo* AcChoR to structural and functional characteristics of AcChoRs from other species and would possibly shed light on the structural and functional basis for myasthenia gravis, which is due to an autoimmune response against the neuromuscular AcChoR (17, 18).

*Torpedo* (a marine elasmobranch) and *Electrophorus* (a freshwater teleost) are highly diverged species whose evolution arose separately from the primordial vertebrate stock (≈400 million years) and accordingly the presence of electric organs in these two species is due to convergent evolution. Sufficient AcChoR can be isolated from both animals to conduct structural analyses of their constituent subunits.

In this study we describe the isolation and amino-terminal sequence determination of the full subunit complement of *Electrophorus* electroplax AcChoR. The structural information obtained is compared with what is known regarding the structure of *Torpedo* AcChoR, the implications of such structures with respect to function are discussed, and a genealogical analysis of the evolution of the polypeptides that comprise the subunits of both AcChoRs is presented.

## MATERIALS AND METHODS

**Preparation of AcChoRs.** Purified, solubilized AcChoR preparations were obtained from *Electrophorus electricus* or *Torpedo californica* electric organ, by using *Naja naja siamensis* $\alpha$-neurotoxin coupled to Sepharose 6B (Pharmacia) as an affinity resin (19).

The specific activity of the purified AcChoR, expressed as nmol of $\alpha$-bungarotoxin binding sites per mg of protein, ranged between 4.3 and 7.1 nmol/mg for *Electrophorus* AcChoR [measured by using a radioimmunoassay (11)] and 8 and 10 nmol/mg for *Torpedo* AcChoR [measured by using a DEAE disk assay (20)]. Morphology of purified AcChoR was studied by negative staining with 1% uranyl acetate.

**Purification of *Electrophorus* AcChoR Subunits.** The purified AcChoR in 10 mM phosphate buffer (pH 7.4) containing 0.2% cholate, 0.5 M NaCl, and 10% glycerol was made 1.5% in $NaDodSO_4$ and incubated at room temperature for 5 min to achieve complete dissociation of the subunits. The denatured AcChoR was dialyzed 1–2 hr at 4°C against 31 mM Tris·HCl (pH 5.8) containing 1.5% $NaDodSO_4$. The dialyzed sample was

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Abbreviation: AcChoR, acetylcholine receptor.

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made 5% in glycerol, 2.5% in mercaptoethanol, 0.002% in bromophenol blue, and 0.2% in sodium thioglycollate. The sample was loaded on a slab gel, prepared according to Laemmli (21), containing 8.75% polyacrylamide. As standards, *T. californica* AcChoR and the Bio-Rad low molecular weight protein $NaDodSO_4$ standards were used. The dimensions of the slabs were: 0.1 × 9 (length) × 13 (width) cm for the running gel and 0.1 × 1.5 × 13 cm for the spacer gel. The gels were run overnight at 5 mA per gel, stained for 2 hr in 0.25% Coomassie brilliant blue/50% methanol/7.5% acetic acid, destained overnight in 20% methanol/7.5% acetic acid, and washed in distilled water at 4°C with many changes for 4–8 hr. The stained protein bands were cut and stored frozen.

The peptides were recovered from the gel by electroelution. The gel strips were chopped into 0.5-mm cubes and incubated overnight at room temperature in 2% $NaDodSO_4$/0.4 M Tris acetate, pH 8. Ten microliters of 10% dithiothreitol was added, and the peptides were electroeluted by using 50 mM Tris acetate buffer (pH 7.8) containing 0.1% $NaDodSO_4$ at 80 V at 4°C for 3–4 days. The efficiency of the elution was monitored by following the parallel elution of an $^{125}I$-labeled subunit of *Torpedo* AcChoR. The eluted samples were desalted by electrodialysis at 4°C for 24 hr at 8 V by using 50 mM ammonium bicarbonate/0.05% $NaDodSO_4$. The $NaDodSO_4$ used in the buffers for electroelution and desalting had been recrystallized twice from hot ethanol. The purity and the integrity of the eluted and desalted samples were checked by $NaDodSO_4$/polyacrylamide gel electrophoresis. The protein bands were visualized by the silver staining method (22).

**Amino-Terminal Amino Acid Sequence Analysis.** The purified subunit samples were lyophilized, dissolved in 30 μl of distilled water, and submitted to amino-terminal sequence analysis by automated Edman degradation on either a spinning cup (23) or a gas phase (24) sequenator. Phenylthiohydantoin-derivatized amino acids were identified by HPLC on an IBM Cyano column. Details on identification of phenylthiohydantoin-derivatized amino acids and standard chromatograms have been described (25).

## RESULTS

The purity of *Electrophorus* AcChoR was assessed by two methods. The first involved determination of the specific activity

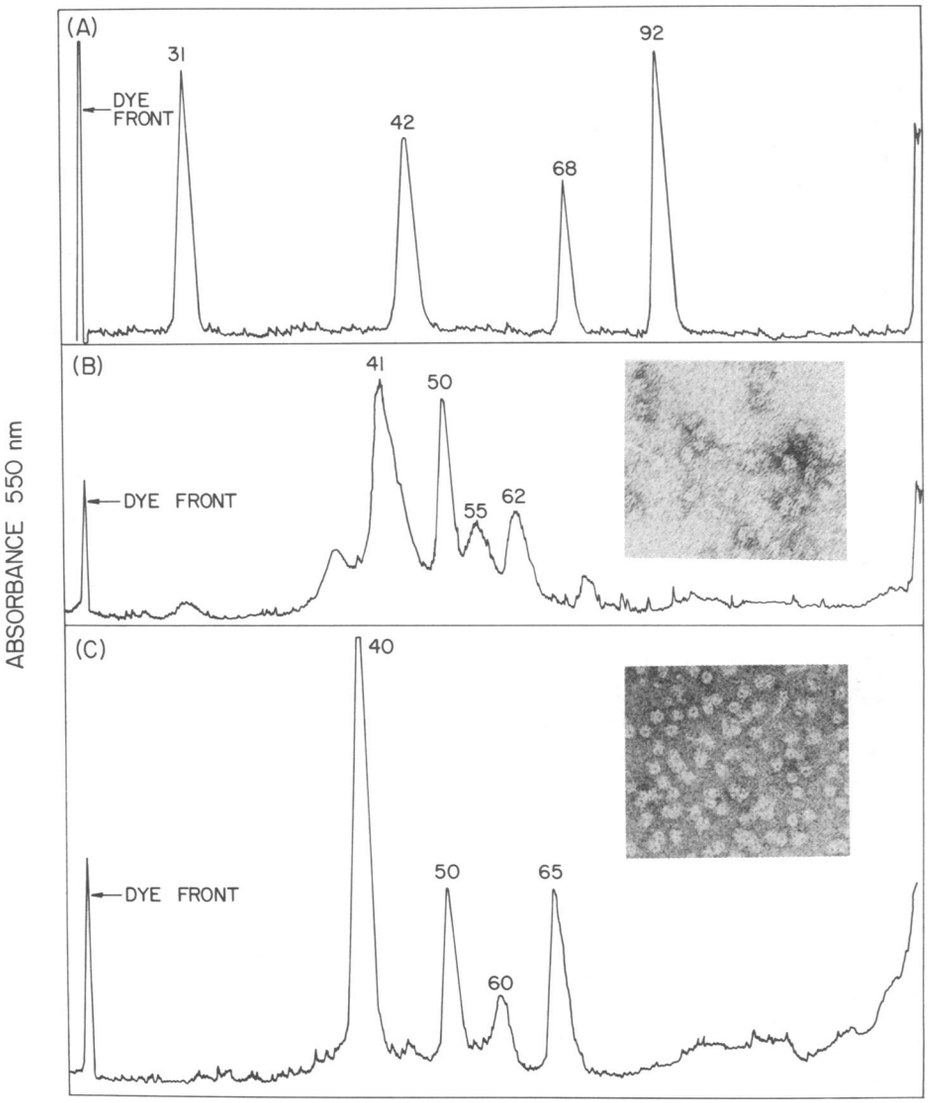


FIG. 1. $NaDodSO_4$ gel electrophoresis scans of purified *E. electricus* (*B*) and *T. californica* (*C*) AcChoR. In *A*, Bio-Rad low molecular weight standards (phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase) are shown. All gels were stained with Coomassie blue. Numbers shown are $M_r \times 10^{-3}$. Electron micrographs of purified AcChoRs from *Electrophorus* (*B Inset*) and *Torpedo* (*C Inset*) visualized by negative staining are shown. (×187,500.)

(nmol of $^{125}$I-labeled $\alpha$-bungarotoxin bound per mg of protein) and the values obtained ranged between 4.3 and 7.1 nmol/mg for various preparations compared with standard *Torpedo* AcChoR (8–10 nmol/mg). The lesser degree of purity for *Electrophorus* AcChoR preparations was also evident in NaDodSO$_4$ gel electrophoresis profiles as shown in Fig. 1. *Torpedo* AcChoR analyzed in this fashion was composed of four polypeptides of $M_r$ 40,000, 50,000, 60,000, and 65,000 (1) with little evidence of contaminating proteins. *Electrophorus* AcChoR preparations were composed mainly of four polypeptides of $M_r$ 41,000, 50,000, 55,000, and 62,000, similar to *Torpedo* subunits. However, other peptides were present in these preparations. Peptides of $M_r$ ≤38,000 increased with aging of the preparation, with a concomitant decrease in the staining intensity of the higher $M_r$ subunits; they were considered to be breakdown products of the AcChoR subunits of higher $M_r$. Two contaminants of $M_r$ >65,000 were consistently present. One of these ($M_r \approx 66{,}000$) was frequently present in amounts roughly corresponding to the levels of the AcChoR subunits, as judged by Coomassie staining intensities. Amino-terminal amino acid sequence analysis of this component yielded the partial sequence

1 5 10

- - LEQKA - GH·····,

which is unrelated to the primary structural data obtained for the homologous AcChoRs polypeptides (see below).

**Electron Microscopy.** Negatively stained preparations of purified *Electrophorus* and *Torpedo* AcChoRs showed the presence of rosette-like structures, with an average diameter of ≈9 nm and an electron-dense central pit (Fig. 1, insets), which have been shown to correspond to individual AcChoR molecules (1).

**Amino-Terminal Amino Acid Sequence.** For each of the four AcChoR subunits, the amino-terminal amino acid sequence was determined for the first 24 amino acid residues. The sequences obtained are reported in Fig. 2. Each of the four subunits represents a single polypeptide chain, because yields at the first step for each of the subunits were 70–90%. These high initial yields eliminate the possibility that a polypeptide with a blocked amino terminus could be present in amounts stoichiometric with the AcChoR subunits. In addition, contaminating sequences were not present at a detectable level (<5%). Minor contamination (10–15%) of the sequences from the higher $M_r$ subunits was occasionally observed, possibly due to the presence of degradation products comigrating in NaDodSO$_4$ gel electrophoresis. The four subunits have distinct but homologous sequences (Fig. 2) and the degree of identity between pairs of subunits (Table 1) ranged between 37.5% and 50%. In 7 of the first 24 positions all four subunits had the same amino acid residue. At numerous other positions (dotted circles in Fig. 2) conservative amino acid substitutions were evident.

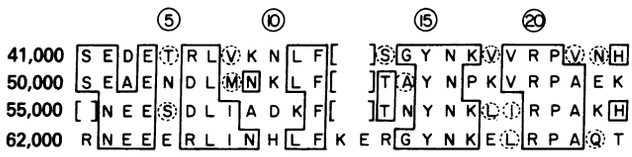


FIG. 2. Amino-terminal amino acid sequences of the four homologous subunits of $M_r$ 41,000, 50,000, 55,000, and 62,000 of *Electrophorus* AcChoR.

In Fig. 3 comparison is made between the amino-terminal sequences of *Torpedo* and *Electrophorus* AcChoR subunits of comparable $M_r$. The extent of sequence identity is indicated in the figure and is summarized in Table 2. Although all subunits from both species are homologous polypeptides, the greatest level of identity in all cases was with the corresponding subunit from each species—i.e., "$\alpha$" to "$\alpha_1$", etc., in terms of the commonly used notation of $\alpha$, $\beta$, $\gamma$, $\delta$ for the *Torpedo* subunits of $M_r$ 40,000, 50,000, 60,000, and 65,000. In 10 of the first 24 positions all the subunits of the AcChoR from both species had the identical amino acid residue (4 positions; residues 4, 7, 15, and 21) or either of two amino acid residues (6 positions; residues 11, 12, 16, 17, 20, and 22).

**Subunit Stoichiometry.** Simultaneous amino acid sequence analysis of the peptides in preparations of intact *Electrophorus* AcChoR was used to determine the subunit stoichiometry. The procedure was similar to that used for quantitation of the subunit composition of *Torpedo* AcChoR (3, 4). Relative amounts of the subunits could be determined from quantitation of the phenylthiohydantoin-derivatized amino acids (cycles 8 and 14). The results from two AcChoR preparations are shown in Table 3. These data yield molar ratios of 2:1:1:1 for the subunits of $M_r$ 41,000, 50,000, 55,000, and 62,000, respectively.

## DISCUSSION

The data we report here demonstrate that *Electrophorus* AcChoR is a pentameric complex of four different subunits, one of which ($\alpha_1$) is present in two copies in the AcChoR complex. The subunits are structurally related, thus allowing the formation of a pseudosymmetric supramolecular complex from four different polypeptides, conforming to the generality that complex protein systems are often constructed from identical or related subunits (26). From the apparent $M_r$ of the four subunits and their 2:1:1:1 stoichiometry, a $M_r$ of 249,000 can be calculated for the *Electrophorus* AcChoR complex; this value fits with experimental determinations obtained with different approaches (1) and is consistent with the size of *Electrophorus* AcChoR as determined by electron microscopy (1).

The high degree of amino acid sequence homology between *Torpedo* and *Electrophorus* AcChoRs demonstrates that the receptor molecule has been highly conserved throughout ani-

Table 1. *Electrophorus* and *Torpedo* AcChoRs: % internal homology (considering only until position 24)

| *Electrophorus* | | | | | *Torpedo* | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Subunit, $M_r$ | 41,000 | 50,000 | 55,000 | 62,000 | Subunit, $M_r$ | 40,000 | 50,000 | 60,000 | 65,000 |
| | | (12) | (9) | (11) | | | | | |
| 41,000 | — | 50 | 37.5 | 46 | 40,000 | — | 37.5 | 37.5 | 50 |
| | | | (10) | (11) | | | | | |
| 50,000 | 50 | — | 42 | 46 | 50,000 | 37.5 | — | 29 | 33 |
| | | | | (12) | | | | | |
| 55,000 | 37.5 | 42 | — | 50 | 60,000 | 37.5 | 29 | — | 50 |
| 62,000 | 46 | 46 | 50 | — | 65,000 | 50 | 33 | 50 | — |

Numbers in parentheses are numbers of identical amino acids.

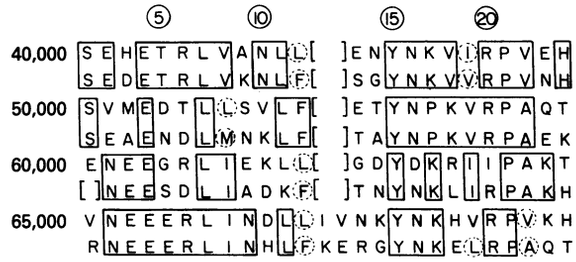


FIG. 3. Comparison of the subunits of comparable $M_r$ ($\alpha$, ≈40,000, $\beta$, ≈50,000, $\gamma$, 55,000 to 60,000, and $\delta$, ≈65,000) from *T. californica* and *E. electricus* electroplax. In each case the sequences of *Torpedo* AcChoR are on the top of each pair.

mal evolution. The similarity in the primary structure of AcChoR subunits from both species explains the difficulties encountered in the past in obtaining antisera specific for the individual subunits; some degree of crossreaction with other subunits was consistently obtained, particularly in the case of the $M_r$ 60,000 and 65,000 subunits, for which "specific" antisera raised in different laboratories consistently showed high cross-reactivity (19, 27–29). Even monoclonal antibodies were found frequently to crossreact with more than one subunit (19, 30). The high degree of identity (up to 71%) between corresponding AcChoR subunits of *Torpedo* and *Electrophorus* explains the extensive crossreactivity of antisera (19, 28) and monoclonal antibodies (19, 30) raised against the subunits of either of these AcChoRs.

The sequence homology alignment in the region investigated in the studies described here required a two-residue insertion in the $M_r$ 64,000 subunit. The homology suggests that the genes encoding each of the four subunits descended from a single ancestral coding sequence. A genealogical tree showing the evolutionary pathway by which the four contemporary subunits of both *Electrophorus* and *Torpedo* AcChoRs can be generated from a single ancestral sequence via minimum nucleotide substitution is shown in Fig. 4.

The shared ancestry and the high degree of conservation of the AcChoR from two evolutionarily distant species suggests the possibility that the AcChoR from most, if not all, vertebrates will have a structure similar to that of the receptors from *Torpedo* and *Electrophorus*. In this respect, there are preliminary indications that mammalian muscle AcChoR also has the same subunit structure as electric organ AcChoR. These include: (*i*) the subunit composition, which, despite the variability reported (1), seems to comprise a major subunit of $M_r$ 40,000 to 45,000 labeled by the same affinity reagents that label the $\alpha$ chain of *Torpedo* and *Electrophorus*, in addition to the occurrence of

Table 2. *Electrophorus* and *Torpedo* AcChoRs: % homology between subunits

| | *Torpedo* | | | |
|---|---|---|---|---|
| *Electrophorus* | 40,000 | 50,000 | 60,000 | 65,000 |
| 41,000 | 17/24<br>71 | 9/24<br>37.5 | 7/24<br>29 | 12/24<br>50 |
| 50,000 | 10/24<br>42 | 13/24<br>54 | 7/24<br>29 | 9/24<br>37.5 |
| 55,000 | 11/24<br>46 | 8/24<br>33 | 11/24<br>46 | 13/24<br>54 |
| 62,000 | 9/24<br>37 | 11/24<br>46 | 12/24<br>50 | 14/26<br>54 |

Table 3. Electric organ AcChoR subunit stoichiometry

| Subunit, $M_r$ | Residues | Preparation* 1 | Preparation* 2 | Average of 1 and 2 |
|---|---|---|---|---|
| 41,000 | Val-8, Gly-14 | 1.90 ± 0.19 | 1.96 ± 0.18 | 1.93 ± 0.19 |
| 50,000 | Met-8, Ala-14 | 1.02 ± 0.02 | 0.99 ± 0.01 | 1.01 ± 0.02 |
| 55,000 | Ala-8, Tyr-14 | 1.10 ± 0.27 | 1.04 ± 0.12 | 1.07 ± 0.20 |
| 62,000 | Ile-8, Glu-14 | 1.02 ± 0.07 | 1.00 ± 0.05 | 1.04 ± 0.06 |

* Values are means ± SEM.

two or three polypeptides of greater mass whose $M_r$ are generally in the same range as electric organ AcChoR; (*ii*) the presence of antigenic determinants common to each one of *Torpedo* AcChoR subunits (32); (*iii*) the close similarity in size and shape: all of the AcChoRs, from *Torpedo* to mammals, appear as annular structures with a diameter of 95 Å and an electron-dense central pit (1, 32).

The remarkable correspondence between subunits of similar $M_r$ (Table 2 and Fig. 3) raises the possibility that each subunit performs a discrete, precise function in AcChoRs from different species. In addition, the close similarity in the primary structure suggests the possibility that the small difference in apparent $M_r$ upon $NaDodSO_4$ gel electrophoresis between corresponding subunits in *Torpedo* and *Electrophorus* (Fig. 1) could be due to different degrees of glycosylation rather than to differences

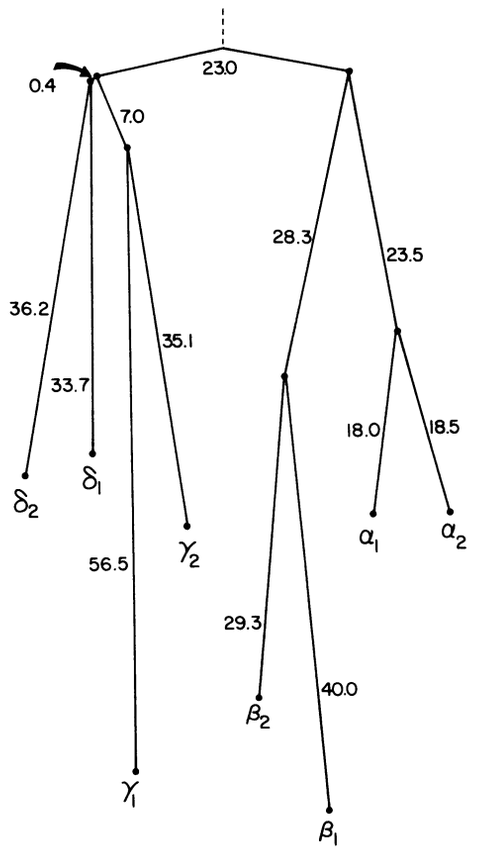


FIG. 4. A phylogenetic tree generated from the amino-terminal sequence data of the four AcChoR subunit types from *T. californica* ($\alpha_1$, $\beta_1$, $\gamma_1$, $\delta_1$) and *E. electricus* ($\alpha_2$, $\beta_2$, $\gamma_2$, $\delta_2$) by using the best fit matrix method (31). Each branch point represents a nodal or ancestral sequence. The numbers associated with each branch length represent the "accepted point mutations" (PAMs) per 100 amino acid residues that occurred in generating the contemporary subunits of both *Torpedo* and *Electrophorus* AcChoRs.

in the actual $M_r$ of the polypeptides. Whether AcChoR subunits also exhibit internal homology units and, if so, whether these are related to the differences in $M_r$ of the subunits remains to be determined.

So far it has been possible to correlate subunit composition and full physiological functionality for *Torpedo* AcChoR (5, 6, 16) but not for *Electrophorus* AcChoR. Because the *Torpedo* AcChoR complex contains both the binding site for acetylcholine and the cation gating unit (5, 6, 16), it has been debated which subunit forms the cation channel. In this respect, the fact that all four subunits of *Torpedo* AcChoR are transmembrane proteins (33, 34), that they are structurally related and arranged in a pseudosymmetrical fashion, and that they form a cylindrical structure containing an indentation in the center argues in favor of the possibility that more than one subunit—possibly most or even all of them—participate in forming a central cation channel.

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